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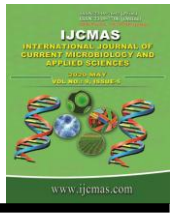

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Evaluation of Sperm Motility with Glutathione and Honey in Skim Milk based Extenders by CASA in Boer Buck

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A B S T R A C T

The aim of the study was to evaluate and compare Boer buck sperm motility with 5 mM Glutathione (G) and or 1% or 2% Honey (H) in Skim milk (SM) based extenders preserved at refrigeration temperature at 0, 24, 48 and 72 hrs. A total of 72 ejaculates were collected equally from 6 mature bucks at weekly interval by using Artificial Vagina (AV) as per standard procedure. All the ejaculates were diluted using six Skim milk based extenders *viz.* SME, SMGE, SMGH(1%)E, SMGH(2%)E, SMH(1%)E and SMH(2%)E. The sperm motility was evaluated by CASA (Computer Assisted Semen Analyzer). The data obtained was statistically analyzed by General Linear Model (GLM) and procedure. The results showed that sperm motility was differed significantly ($P<0.05$) from extender to extender at 24 h, 48 h and 72 h of refrigeration. Based on the results concluded that supplementation of optimum concentration of glutathione (5 mM) and honey (1%) maintained better sperm motility upto 72 hours of storage and successful preservation of buck spermatozoa at refrigeration temperature. Hence study revealed that Boer buck semen can be preserved effectively with SMGH(1%)E, at refrigeration temperature upto 72 hours of storage.

Introduction

Five home-made Skim-milk extenders like cow skim-milk (CSM), goat skim-milk (GSM), sheep skim-milk (SSM), buffalo skim-milk (BSM) and commercial dried skim-milk (CDSM) were examined for the motility, morphology, plasma membrane integrity and viability of sperms at 0, 24, 48, 72, 96 and 120 hours respectively (Rimon *et al.,* 2017). Removal of seminal plasma improved the viability and longevity of Beetal buck spermatozoa in skimmed milk based extenders during cooling upto 72 hours (Hassan *et al.*, 2016).

Generally sperm motility is expressed in terms of mass motility and progressive motility. Conventional methods for measuring the sperm kinematic characters is difficult, time consuming and subjective. To get over these difficulties Computer Assisted Semen

Analysis (CASA) is the equipment of choice to provide precise and accurate information on sperm motion characteristics. The importance of analyzing kinematics and motility patterns of spermatozoa were studied using CASA (Anand *et al.,* 2016).

The sperm motility, viability and fertilizing ability can be improved or preserved by the addition of various motility enhancing agents or antioxidants in semen diluents. These antioxidants can neutralize or reduce the risk of damage to spermatozoa by activating antioxidant enzymes (Glutathione peroxidase - GSH and Catalase) during cryopreservation process or combating harmful effects of ROS. Glutathione plays a cofactor role for glutathione peroxidase (GSH) which in turn reduces hydrogen peroxide (H_2O_2) to H_2O and also lipoperoxides to alkyl alcohols (Noei *et al.,* 2015). Glutathione and Vitamin-E improved the buck seminal parameters during hypothermic storage of liquid semen at 4^0C for 72 hours (Sarangi *et al.,* 2017). Honey bee (HB) has a potent antioxidant and antibacterial properties (Zoheir *et al.,* 2015). At low temperatures honey does not freeze and its viscosity increases with decreasing the temperature. This creates a low surface tension that eventually minimizes the formation of ice crystals inside the cytoplasm of sperm and hence reduces damage to the spermatozoa during cryopreservation (El-Sheshtawy *et al.,* 2014).

Years to come with supplementation of an agent in the extender that can overcome the threat of antibiotic resistance and at the same time would maintain the motility of sperm by taking care of heavy bacterial load in the buck's semen to prove a very useful strategy for successful preservation **(**Banday *et al.,* 2017). The wide property of honey and its addition into freezing medium is also expected for the protection of spermatozoa during cryopreservation. To this honey as a natural product and has been studied as supplement with different properties like synergistic antioxidant, non-permeant cryoprotectant and energy source for the improvement of post-thaw semen quality (Yimer *et al*., 2015).

Materials and Methods

The study was carried out in six sexually mature Boer bucks maintained at Department of Veterinary Gynaecology and Obstetrics, Veterinary College, Bidar. All the bucks were maintained under uniform conditions and reared under semi-intensive housing system. Bucks were kept in a single flock with routine deworming and vaccination as per schedule. The bucks were allowed for free grazing for 5-6 hrs daily, fed concentrate @ 250 g per day per animal and provided *ad libitum* drinking water throughout the day.

Preparation of honey solution

1 mL of honey was added to 9 mL double distilled water (v/v) to prepare a total concentration of 10% (10 ml) honey solution (El-Sheshtawy *et al*., 2014). Out of 10 ml (10%, v/v) honey solution the volume of 1 ml and 2 ml having of 1% (v/v) and 2% (v/v) concentrations respectively were used.

Semen collection

A total of 72 ejaculates were collected equally from 6 mature bucks at weekly interval by using Artificial Vagina (Fig. 1) as per standard procedure.

Mass activity

A drop (0.5 µL) of neat semen was placed on a pre-warmed glass slide (37^0C) without cover slip and examined under phase contrast microscope (100 X) with low power magnification (100 X). Wave motion

characteristics or swirl motion of the spermatozoa were scored (Table 1) from + to ++++ scale (Blokhuis, 1962).

Sperm motility

It was assessed using a phase contrast microscope (400 X magnification) with a warm stage maintained at 37^0C. A wet semen mount was made using a small drop of semen placed directly on a microscope slide covered by a cover slip (Loskutoff and Crichton, 2001) and sperm motility was scored (Table 2).

Semen dilution

The collected ejaculates were centrifuged @ 1,500 rpm for 10 minutes and supernatant seminal plasma was discarded. The sperm pellets (Fig. 2) washed with isosmotic phosphate buffered saline (PBS) solution in equal volume to obtain an optimal concentration and pH for the sample. Then buck wise sperm pellet sample was diluted with 6 (Table 3) different extenders (1:60) stored at refrigeration temperature and thawed to room temperature (37^0C) in a water bath at the time of semen evaluation.

Evaluation of semen by computer assisted semen analysis (CASA)

All the diluted sperm pellet samples were preserved at refrigeration temperature and thawed to room temperature (37^0C) in a water bath at the time of evaluation by Computer Assisted Semen Analysis system using model Biovis - CASA 2000 (Expert Vision labs Pvt. Ltd. Mumbai, India) at 0, 24, 48 and 72 hours of storage.

CASA procedure

A drop of diluted semen was taken on a clean grease free slide which was covered by a cover slip and it was focussed under phase contrast microscope with 100 X magnification. Biovis-CASA software was turned on, fine adjustment was made for viability and clicked on option capture which captured around 60 frames/minute then automatically analysed for sperm concentration, motility and other mentioned parameters (Fig. 3). Assessment of diluted chilled semen by CASA was performed at 24, 48 and 72 h of storage.

Statistical analysis

The data obtained was statistically analyzed by General Linear Model (GLM) and procedure using SAS - Statistics Version 9.3, SAS Inc., Cary, NC; 2010 software.

Results and Discussion

Semen evaluation tests in Boer bucks

The volume of neat semen ranged from 0.81 to 1.30 ml having whitish yellow color. The mass activity was ++++ scale for all semen samples and sperm motility ranged from 91.66 ± 1.12% to 96.66 ± 1.42% and none of the samples has shown presence of any foreign body (Table 4 and Figure 1).

Comparative effect of Boer buck sperm motility with 5 mM glutathione and or 1% or 2% honey in skim milk based extenders preserved at refrigeration temperature at 0, 24, 48 and 72 h

Motile sperm percentage

The motile sperm percentage varied from extender to extender with significant ($P<0.05$) difference at 24 h, 48 h and 72 h of refrigeration. Addition of 1% honey in skim milk glutathione extender increased motile sperm percentage while addition of 1% honey in skim milk extender reduced it at 72 h of refrigeration (Table 5 and Figure 2).

Immotile sperm percentage

The values of immotile sperm percentage differed significantly (P<0.05) from extender to extender at 24 h, 48 h and 72 h of refrigeration. Addition of 1% honey in skim milk glutathione extender reduced immotile sperm percentage in contrast addition of 2% honey in skim milk extender increased it at 72 h of refrigeration (Table 6 and Figure 3).

Discussion

Semen evaluation tests in Boer bucks

The volume of neat semen ranged from 0.81 to 1.30 ml having whitish yellow color. The mass activity was ++++ scale for all semen samples and sperm motility ranged from 91.66 ± 1.12% to 96.66 ± 1.42% and none of the samples has shown presence of any foreign body (Table 4 and Figure 1). Similar to the present study Kumbari (2017) recorded the semen volume ranged from 0.8 ± 0.1 to 1.2 ± 0.2 mL while the color and mass activity were whitish - yellow and ++++ scale respectively in Boer bucks. However Florence *et al.*, (2011) recorded the bucks pooled ejaculate average volume of 2.5 ml, creamy color and homogenous without flakes or clumps with average gross motility and sperm motility were assessed as +++ scale and 95% respectively.

Comparative effect of Boer buck sperm motility with 5 mM glutathione and or 1% or 2% honey in skim milk based extenders preserved at refrigeration temperature at 0, 24, 48 and 72 h

Motile sperm percentage

The motile sperm percentage varied from extender to extender with significant (P<0.05) difference at 24 h, 48 h and 72 h of refrigeration. Addition of 1% honey in skim milk glutathione extender increased motile sperm percentage while addition of 1% honey in skim milk extender reduced it at 72 h of refrigeration (Table 5 and Figure 2). Kadaganchi (2017) also concluded that values for motile sperm percentage varied from dilutor to dilutor with significant difference at 0, 24, 48 and 72 hours of preservation. The motile sperm percentage was recorded higher in SMFE and lower in SMFH(2%)GE at 0, 48 and 72 hours, however, SMFH(2%)E dilutor shown less motile sperm percentage at 24 hours without any significant difference. The motile sperm percentage declined with increase in holding time in all extenders (p<0.05). In contrast, Nancy (2018) stated that there was no effect of addition of antioxidants, almond and olive oil (0.25%) in the semen extenders on motile sperm percentage.

Immotile sperm percentage

The values of immotile sperm percentage differed significantly (P<0.05) from extender to extender at 24 h, 48 h and 72 h of refrigeration. Addition of 1% honey in skim milk glutathione extender reduced immotile sperm percentage in contrast addition of 2% honey in skim milk extender increased it at 72 h of refrigeration (Table 6 and Figure 3). Similarly, Kadaganchi (2017) reported that, values for immotile sperm percentage varied from dilutor to dilutor with significant difference at 0, 24 48 and 72 hours of semen storage. The immotile sperm percentage improved (p<0.05) with increase in holding time in all extenders. As against, Nancy (2018) stated that, immotile sperm percentage increased (P <0.05) with increase in holding time in all extenders but there was no effect of addition of almond and olive oil (0.25%) as antioxidants to reduce immotile sperm percentage.

Table.1 Evaluation of semen for mass activity

Mass activity	Score
++++	Rapid cloud formation
+++	Good cloud formation
++	Strong flow with some thickening
+	Poor flow

Table.2 Score chart for assessing sperm motility of the semen samples

Sperm motility	Percentage (%)
No movement	0
No forward progression (only head movement)	1 - 20
Slow forward sperm progression (usually with laboured head movement)	20 - 40
Fast forward sperm progression	40 - 60
Faster forward sperm progression	60 - 80
Very fast forward movement	80 - 100

Table.3 Group wise semen extenders with abbreviations

Group No.	Semen Extenders	Abbreviations
I	Skimmed Milk Extender	SME
II	Skimmed Milk - Glutathione Extender	SMGE
III	Skimmed Milk - Glutathione - Honey (1%) Extender	SMGH(1%)E
IV	Skimmed Milk - Glutathione - Honey (2%) Extender	SMGH(2%)E
V	Skimmed Milk - Honey (1%) Extender	SMH(1%)E
VI	Skimmed Milk - Honey (2%) Extender	SMH(2%)E

Table.4 Semen evaluation tests in Boer bucks

Buck No.	Volume (mL) (Mean ± SE)	Colour	Mass activity (+ to ++++)	Sperm motility (%) (Mean ± SE)
1	1.02 ± 0.04	Whitish - yellow	++++	94.16 ± 1.48
2	0.82 ± 0.04	Whitish - yellow	++++	92.50 ± 1.30
3	0.91 ± 0.04	Whitish - yellow	++++	93.33 ± 1.42
4	0.81 ± 0.02	Whitish - yellow	++++	91.66 ± 1.12
5	1.30 ± 0.05	Whitish - yellow	++++	96.66 ± 1.42
6	1.21 ± 0.04	Whitish - yellow	++++	95.83 ± 1.48

Table.5 Motile sperm percentage (Mean ± SE) in sperm pellets diluted with skim milk based extenders at 0, 24, 48 and 72 h of storage at refrigeration temperature

Extenders	0 h	24 h	48 h	72 h
SME	$93.95^{aABC} \pm 0.64$	$87.66^{bAC} \pm 0.60$	$71.21^{cA} \pm 0.76$	$60.09^{dA} \pm 0.99$
SMGE	$93.05^{aAC} \pm 0.82$	$81.70^{bB} \pm 0.96$	$64.86^{cB} \pm 0.82$	$56.05^{dAC} \pm 1.01$
SMGH(1%)E	$95.95^{aB} \pm 0.51$	$88.29^{bA} \pm 0.35$	$83.04^{cC} \pm 0.39$	$76.80^{dB} \pm 0.65$
SMGH(2%)E	$94.65^{aCB} \pm 0.65$	$87.85^{bAC} \pm 0.35$	$78.67^{cDE} \pm 0.50$	$74.34^{dB} \pm 0.27$
SMH(1%)E	$94.65^{aCB} \pm 0.59$	$88.40^{bA} \pm 0.47$	$75.35^{cE} \pm 1.65$	$59.82^{dA} \pm 1.02$
SMH(2%)E	$94.21^{aCB} \pm 0.54$	$85.62^{bC} \pm 0.73$	$76.77^{cE} \pm 0.78$	$52.51^{dC} \pm 1.56$

Note: SME: Skim Milk Extender, SMGE: Skim Milk Glutathione Extender, SMGH(1%)E: Skim Milk Glutathione Honey (1%) Extender, SMGH(2%)E: Skim Milk Glutathione Honey (2%) Extender, SMH(1%)E: Skim Milk Honey (1%) Extender, SMH(2%)E: Skim Milk Honey (2%) Extender
Means with different superscripts differ significantly at P<0.05
^{abcd} superscripts indicated the difference between time (columns) within extenders (rows)
^{ABCDE} superscripts indicated the difference between extenders (rows) within time (columns)

Table.6 Immotile sperm percentage (Mean ± SE) in sperm pellets diluted with skim milk based extenders at 0, 24, 48 and 72 h of storage at refrigeration temperature

Extenders	0 h	24 h	48 h	72 h
SME	$6.04^{aABC} \pm 0.64$	$12.33^{bAC} \pm 0.60$	$28.78^{cA} \pm 0.76$	$39.91^{dA} \pm 0.99$
SMGE	$6.94^{aAC} \pm 0.82$	$18.30^{bB} \pm 0.96$	$35.13^{cB} \pm 0.82$	$43.94^{dAC} \pm 1.01$
SMGH(1%)E	$4.04^{aB} \pm 0.51$	$11.70^{bA} \pm 0.35$	$16.95^{cC} \pm 0.39$	$23.20^{dB} \pm 0.65$
SMGH(2%)E	$5.34^{aCB} \pm 0.65$	$12.15^{bAC} \pm 0.35$	$21.32^{cDE} \pm 0.50$	$25.65^{dB} \pm 0.27$
SMH(1%)E	$5.34^{aCB} \pm 0.59$	$11.60^{bA} \pm 0.47$	$24.64^{cE} \pm 1.65$	$40.17^{dA} \pm 1.02$
SMH(2%)E	$5.78^{aCB} \pm 0.54$	$14.37^{bC} \pm 0.73$	$23.22^{cE} \pm 0.78$	$47.48^{dC} \pm 1.56$

Note: SME: Skim Milk Extender, SMGE: Skim Milk Glutathione Extender, SMGH(1%)E: Skim Milk Glutathione Honey (1%) Extender, SMGH(2%)E: Skim Milk Glutathione Honey (2%) Extender, SMH(1%)E: Skim Milk Honey (1%) Extender, SMH(2%)E: Skim Milk Honey (2%) Extender
Means with different superscripts differ significantly at P<0.05
^{abcd} superscripts indicated the difference between time (columns) within extenders (rows)
^{ABCDE} superscripts indicated the difference between extenders (rows) within time (columns)

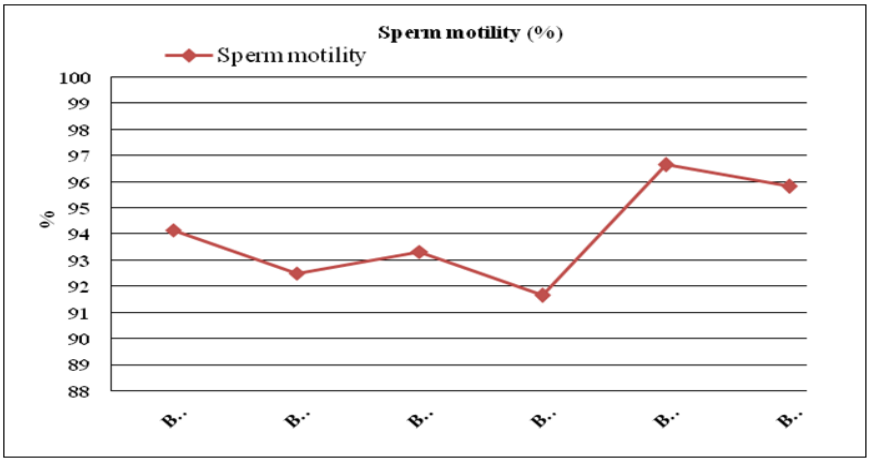

Figure.1 Semen evaluation tests in Boer bucks

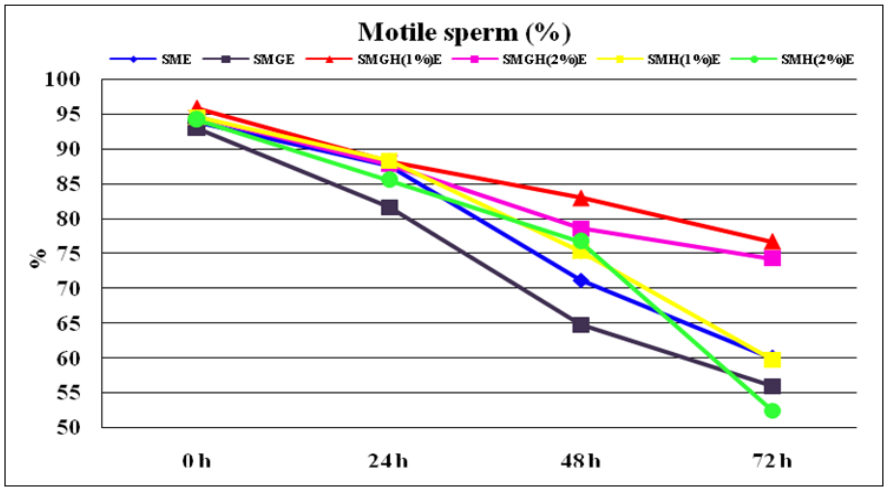

Figure.2 Motile sperm percentage in sperm pellets diluted with skim milk based extenders at 0, 24, 48 and 72 h of storage at refrigeration temperature

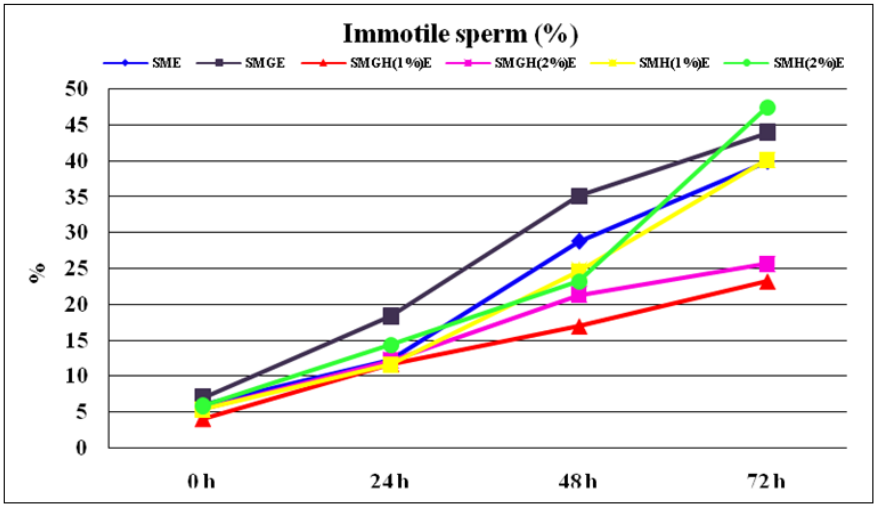

Figure.3 Immotile sperm percentage in sperm pellets diluted with skim milk based extenders at 0, 24, 48 and 72 h of storage at refrigeration temperature

The results from the research study showed that both motile and immotile sperm percentages were differed significantly ($P<0.05$) from extender to extender at 24 h, 48 h and 72 h of refrigeration. Based on the results concluded that supplementation of optimum concentration of glutathione (5 mM) and honey (1%) maintained better sperm motility upto 72 hours of storage and successful preservation of buck spermatozoa at refrigeration temperature. Hence Boer buck semen can be preserved effectively with SMGH(1%)E, at refrigeration temperature upto 72 hours of storage.

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